ASSAY METHODS FOR DETECTION OF A VIRUS IN AN AVIAN TISSUE SAMPLE

The present invention relates to assay methods and, in particular, to methods for detecting the presence of a virus, especially Marek's disease virus (MDV), in an avian tissue sample.

Background

Marek's disease virus (MDV) is a herpesvirus, which causes lymphoproliferative disease in chickens. Even after the introduction of vaccines against MDV, the infection still causes considerable losses in the poultry industry. MDV is divided into three serotypes, all of which establish latent infections. Serotype 1 includes oncogenic viruses, serotype 2 non-oncogenic viruses and serotype 3 includes the turkey herpesviruses (HVT) (Bülow *et al* (1976) *Zentralblatt für Veterinarmedizin,* 23B, 391-402).

The traditional diagnosis of Marek's disease is based on the clinical signs and pathological alterations. However, more specific methods for surveillance of the prevalence of MDV would be desirable. The detection of viral antigen in the feather follicle epithelium by the agar gel precipitation test has been described by Haider *et al* (1970) *Poultry Science,* 49, 1654-1657. The different serotypes can be differentiated by the agar gel precipitation test (Lee *et al* (1983) *Journal of Immunology, 130,* 1003-1006), but the sensitivity of that test is inferior to that of enzyme-linked immunosorbent assay (ELISA) and DNA hybridization (Davidson *et al* (1986) In: Current research on Marek's disease. *Proceedings of the 5th International Symposium on Marek's Disease* (pp. 311-316). Tallahassee: Rose Printing Company, Inc.).

The preferred samples for virus isolation are buffy-coat cells, which can be co-cultivated with susceptible primary cell cultures. Immunofluorescent assay (Kitamoto *et al* (1979). *Biken Journal, 4,* 137-142) or ELISA (Cheng *et al* (1984) *Avian Diseases*, 4, 900-911), can be used for subsequent identification of the MDV serotype. Alternatively, the serotype can be identified by restriction endonuclease analysis (Ross *et al* (1983). *Journal of General Virology,* 64, 2785-2790) or polymerase chain reaction (PCR) (Wang *et al (1993) Molecular and Cellular Probes, 7,* 127-131. *In situ* hybridization has been used for detection of MDV genome in infected tissue (Endoh *et al* (1996) *Journal of Veterinary Medical Science*, 58, 969-976; Ross *et al* (1997) *Journal of General Virology,* 78, 2191-2198), but this technique is probably too laborious for routine diagnoses. (Davidson *et al* (1995) *Avian Pathology, 24,* 69-94; and Davidson *et al* (1996) In: Current research on Marek's disease. *Proceedings of the 5th International Symposium on Marek's Disease (*pp. 311-316). Tallahassee: Rose Printing Company, Inc.), applied MDV serotype 1-specific PCR techniques to full blood and tumour tissue samples from commercial chicken and turkey flocks, the majority of which had neoplastic disease. Wang *et al* (1993) *Molecular and Cellular Probes*, 7, 127-131; Young, P. & Gravel, J. (1996) In Current *research on Marek's disease. Proceedings of the 5th International Symposium on Marek's Disease* (pp. 308-310),. Tallahassee: Rose Printing Company, Inc.; and Silva, R.F. & Witter R.L. (1996) In *Current research on Marek's disease. Proceedings of the 5th International Symposium on Marek's Disease* (pp. 302-307). Tallahassee: Rose Printing Company, Inc., applied a MDV serotype 1-specific PCR protocol to various tissues of chickens experimentally inoculated with the JM/102 strain.

Handberg *et al* (2001) *Avian Pathology* 30 : 243-249 describe the use of serotype 1- and serotype 3-specific PCR for the detection of MDV in

chickens. Tissue samples were taken from blood (buffy-coat cells), spleen, liver, skin, feather tips and ovaries.

Description of the invention

The present invention provides further methods for detecting a virus, especially MDV, in avian tissue samples.

In a first aspect the invention provides a method of detecting a virus in an avian tissue sample comprising: extracting genetic material from an avian tissue sample; and testing the extracted genetic material to detect any genetic material from the virus; characterised in that the avian tissue sample is derived from one or more feathers of the axillary tract.

By using feather samples the test can be carried out on live animals. Sampling of feathers is simple, quick and practical under field conditions. Feather samples can be placed in a suitable container and tested immediately or stored for future testing, as desired. By contrast, sampling blood requires great care to prevent blood clots forming, including transport of blood under cool, controlled conditions. Blood clotting leads to negative test results. Internal organ samples, such as spleen and tumour samples, must be transported on wet ice, which is impractical under field conditions.

By selecting axillary tract feathers from which to derive a tissue sample, the invention provides significant advantages over known methods which take tissue samples from different parts of the bird.

Surprisingly, we have found that virus can be detected in axillary tract feathers at higher levels than in other feathers and therefore virus can be detected in axillary tract feathers according to the invention when it cannot

be detected in other tissue samples, including other feathers. Accordingly, the methods of the invention are particularly suitable for monitoring the extent to which a flock of birds has been immunised effectively with MDV vaccine, by detecting the presence of the vaccine strain in axillary tract feather tissue samples.

By "avian" we include any bird, but preferably birds which are produced commercially, especially poultry such as chickens, turkeys, ducks, etc.

By "axillary tract feathers", we include the meaning of the feathers located in the region of a bird marked "axillary" in the accompanying feathering diagram (Figure 1). Preferably, the axillary tract feather selected is a "pin feather", that is, an immature growing feather. The term "pin feather" will be familiar to skilled persons. For example, van Tyne J & Berger AJ (1959) *Fundamentals of Ornithology*, John Wiley, New York refer to a pin feather as "a new, growing feather, still not completely unsheathed". Lucas AM & Stettenheim PR (1972) *Avian Anatomy*, Integument Part 1, US Government Printing Office, Washington, pp 199-200 remark "The new feather is tightly furled inside a sheath while it forms. As it appears above the skin, it has a long conical shape with a blunt tip and a slightly moist surface. A feather at this stage in any generation is often called a pin feather". (See also Figure 1(a), which is taken from Lucas & Stettenheim (1972)).

Preferably, the axillary tract feathers are taken from chicks which are advantageously less than a month old.

In a preferred embodiment, the method is performed on samples taken from chicks on or prior to 13 days post-immunisation, preferably at between 8 days to 12 days post-immunisation, more preferably at between 9 to 11 days post-immunisation, i.e. on days 9, 10 or 11 post-immunisation.

Preferably, the method provides quantitative information on the amount of virus, especially MDV, in the sample.

Preferably, the method is specific for MDV serotype 1, and more preferably the method is specific for MDV-1 Rispens strain CVI 988. The latter strain is a commercial vaccine strain produced by Fort Dodge, IA, USA which is available from the American Type Culture Collection (ATCC), Mannassas, VA, USA.

Advantageously, the method involves the use of a PCR reaction. Preferably, before said PCR reaction is carried out, the extracted genetic material to be tested is treated with an agent to overcome the inhibitory effect of any feather tissue factor which may be present. This inhibitory effect appears to be associated with melanin and can therefore be a particular problem when feathers from brown birds are sampled. Preferably, the agent is selected from one or more of bovine serum albumin; porcine (pig) albumin; and ovine (sheep) albumin.

Skilled persons will be aware of a range of detection methods for detecting viral, especially MDV, genetic material which could be used in the methods of the invention, such as the methods of Handberg *et al* (2001) *supra*. A particularly preferred method for detecting MDV-1 strain CVI 988 is as follows:

(i) providing forward and reverse primers for a nucleic acid polymerase, which primers are selected from the nucleotide sequence which flanks the 132 bp repeat nucleotide sequence of MDV;

(ii) amplifying nucleic acid sequences between the primers;

(iii) detecting the number of 132 bp repeat sequences in the amplified nucleic acid sequences; and

(iv) relating the number of 132 bp repeat sequences to the identity of the viral nucleic acid and thereby identifying the type of MDV in the tissue sample, multiple copies of the 132 bp repeat sequence being indicative of MDV-1 strain CVI 988.

A preferred quantitative method for use in detecting MDV according to the present invention comprises:

(a) providing a polynucleotide sequence which is capable of binding specifically to a MDV-specific target polynucleotide;

(b) contacting the extracted genetic material with a probe whereby the probe binds specifically to its target MDV polynucleotide;

(c) determining whether the probe has bound to its target MDV polynucleotide; and

(d) determining whether the sample contains MDV on the basis that the presence of the target polynucleotide indicates the presence of MDV in the sample.

Step (d) preferably provides a quantitative determination of the amount of virus in the sample.

Advantageously the step of determining whether the probe has bound to a target polynucleotide comprises amplifying a region of the target polynucleotide, which region comprises the binding site of the probe.

Preferably the probe has the sequence 5' AGA CCC TGA TGA TCC GCA TTG CGA CT 3' (SEQ ID No. 1).

Preferably, amplification is primed by the following primers:

Forward primer (GGT CTG GTG GTT TCC AGG TGA – SEQ ID No. 2) which is located at NT positions 1341-1361 in the GA strain Meq gene sequence.

The GA (Georgia) strain was a 1964 isolate from Georgia, from an ovarian tumour. Reference: C.S. Eidson & S.C. Schmittle (1968). Studies on acute Marek's disease. I. Characteristics of isolate GA in chickens. Avian Diseases 12;467-476.

Reverse primer (GCA TAG ACG ATG TGC TGC TGA – SEQ ID No. 3) is located at NT positions 1413-1393.

Advantageously, the probe is labelled fluorescently and the step of determining whether the probe has bound to a target polynucleotide comprises determining the fluorescent emissions of the probe.

Two fluorescent dyes are brought into physical proximity by direct conjugation at opposite ends of a short oligo probe (5' reporter fluorochrome, usually 6-FAM, and 3' quencher fluorochrome, usually TAMRA). When the high-energy fluorophore (FAM) is excited at 488 nm, instead of the expected fluorescence emission at 520 nm the captured energy is transferred to the lower energy fluorophore (TAMRA) and is emitted at 580 nm (fluorescence resonance energy transfer, FRET, has occurred). Using the fluorescein/rhodamine reporter/quencher combination, FRET will effectively occur even when the groups are separated by 25-30 bases of DNA. During the course of a TaqMan™ assay the two fluorophores are physically detached from each other by the 5'-nuclease

action of Taq DNA polymerase - after which 488 nm stimulation results in visible FAM emission at 520 nm.

Dual-labeled probes usually have a 5'-reporter dye, such as FAM, TET, HEX, JOE or VIC and a 3'-quencher group, such as TAMRA or Dabcyl (a universal quencher).

Suitable fluorescent labels are within the common general knowledge of skilled persons. The following reagents are available from Sigma-Aldrich, UK: HEX stands for hexachloro-fluorescein; TET stands for tetrachloro-fluorescein; Joe is 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein; ROX is 5-carboxy-Rhodamine; dabcyl is 4-((4-(dimethylamino)phenyl)azo)benzoic acid.

Structures and further information on all of these are available on the molecular probe website www.probes.com. VIC dye is available from Applied Biosystems.

6-FAM = 6-carboxyfluorescein; (a phosphoramidite); yellow-green dye; absorbance maximum = 494 nm, emission maximum = 525nm

HEX = a phosphoramidite; pink dye; absorbance maximum = 535, emission maximum = 556

TET = a phosphoramidite; orange dye; absorbance maximum = 521, emission maximum = 536

VIC = absorbance maximum 538, emission maximum = 554

JOE = absorbance maximum 521, emission maximum = 547

TAMRA = 6-carboxy-tetramethyl-rhodamine; absorbance maximum = 555, emission maximum = 580

Dabcyl = absorbance maximum = 453, no maximum emission (universal quencher)

From the foregoing description it will be apparent that an important aspect of the methods of the invention is the use of avian tissue samples which are more convenient and useful than avian tissue samples used previously for detecting viral, especially MDV, infection. Accordingly, further aspects of the invention relate to the provision of avian tissue samples.

In a second aspect the invention provides an isolated avian tissue sample from one or more feathers from the axillary tract.

By "isolated" we include the meaning that the tissue sample is free of a substantial amount of the material with which it is normally associated in nature. For example, the tissue sample may be stored in a container, or be derived from the original axillary tract feather by a variety of isolation; extraction and/or purification methods.

Preferably the isolated tissue sample consists of the proximal portion (the non-barbed portion which is attached to the skin and which contains the pulp – see accompanying figures). Accordingly it is preferred that the proximal portion of the axillary tract feather is isolated from the distal (barbed) portion of the feather. This is easily achieved simply by cutting off the proximal portion of the feather with a pair of scissors and discarding the distal portion.

In a third aspect the invention provides a genetic material-containing extract from an avian tissue sample wherein the extract is taken from a sample of tissue as described in relation to the second aspect of the invention.

It will be appreciated that samples according to the second and third aspects of the invention may be collected and/or prepared in the field, or transported

to a separate location, such as a laboratory, for preparation and testing. Hence, further aspects of the invention relate to samples according to the second and/or third aspects of the invention stored in a form suitable for transport to a separate location.

The feathers could be stored complete (eg. in 20 ml Sterilin universal plastic tubes), or after cutting off the proximal portion required for DNA preparation (eg. in 1 ml Eppendorf snap-cap or screw-cap tubes). Alternatively, the feathers could be stored in heat-sealed or tied plastic bags. For short-term storage (eg. 1 week), the feathers could be stored at 4^0C, but, for longer periods of storage, they should be stored frozen at -20^0C.

The DNA should be stored at -20^0C in screw-cap or snap-cap 0.5 ml or 1.5 ml Eppendorf tubes, in Tris-EDTA buffer or, if the DNA is to be used in Taqman analysis, in water since EDTA will inhibit the Taqman reaction.

Advantageously, the results of the methods are furnished in an intelligible format. Preferably, the results are recorded or stored on an information carrier. However, the step of furnishing the results could be by communicating the results orally.

By "information carrier", we include any means of storing information, such as paper, a computer disk; an internet-based information transfer system, such as an e-mail or internet page, or electronic file, etc. Of course, an "intelligible format" is also intended to embrace encrypted information which can be deciphered with an approximate key.

Examples embodying certain aspects of the invention will now be described with reference to the following figures in which:

Figure 1(a) shows the development of feathers above the skin, during the first four generations. A circle indicates a feather that is still growing; a dot, a feather that is fully grown.

Figure 1(b) is a diagram of the feathering pattern in chickens which shows the axillary tract.

The axillary tracts lie on the underside of the chick and extend, each side, from the lower neck to the upper abdomen, underneath the wings and alongside the breastbone. Figure 1 is taken from the book 'Bird Structure: An approach through evolution development and function in the fowl'. D.A.Ede, Publisher: Hutchinson Educational, 1964. The feathering pattern is the same in all birds.

Figures 2(a) to 2(d) are photographs of the axillary tract feathers and close ups of individual feathers showing the portions which are retained to provide tissue samples according to the invention. Figures 2(a) and 2(b) demonstrate the axillary tract feathers. Figures 2(c) and 2(d) demonstrate the part of the feather taken for analysis.

Figure 3:

Shows a dose response of serotype CVI988 vaccine as detected by *Meq* primers (78 bp product) with Taqman technique. CVI988 vaccine was harvested from a bacterial artificial chromosome (BAC) vector.

This experiment was performed to test/optimise the use of the *Meq* Taqman primer/probe set. BAC10 DNA was used as the target DNA since it was known to contain many copies of the viral genome.

Taqman assay was used to detect the *Meq* gene in DNA derived from Rispens virus genome cloned into a Bacterial Artificial Chromosome (BAC10). The DNA was used in ten-fold dilutions. During each cycle of real-time PCR, the reporter fluorochrome FAM is released and able to fluoresce. Therefore, with each cycle, fluorescence intensity increases. The Ct value (the cycle at which fluorescence passes a fixed threshold) is a measure of the starting copy number of the target sequence. The lower the Ct value, the higher the starting copy number of target sequence. A Ct value of 40 indicates that no target sequence, or an undetectable amount of target sequence, is present. This figure shows that dilutions of this DNA preparation between 1:10 and 1:10000 gave a detectable amount of Meq PCR product. The Ct value increases linearly with increasingly dilute DNA.

Figure 4:

Dose response of CVI988 vaccine in chicken embryo fibroblasts (CEF) and bacterial artificial chromosomes (BAC). This experiment was performed to test/optimise the use of the *Meq* Taqman primer/probe set on DNA derived from Rispens- MDV infected cells.

Taqman assay was used to detect the *Meq* gene in DNA derived from BAC10, and in DNA derived from Rispens MDV-infected chick embryo fibroblast (CEF) cells. The DNA was used in ten-fold dilutions. This figure shows that for both BAC10 DNA and Rispens-infected CEF DNA, the Ct value increases linearly with increasingly dilute DNA.

Figure 5:

Dose response of CVI988 vaccine in chicken spleen and CEF. This experiment was performed to test/optimise the use of the *Meq* Taqman

primer/probe set on DNA derived from tissue samples from Rispens- MDV infected chickens.

Taqman assay was used to detect the *Meq* gene in DNA from a spleen of a Rispens–inoculated chick (11 dpi), and an age-matched uninoculated chick. DNA from Rispens-infected CEF cells was used as a positive control. The DNA was used in ten-fold dilutions of 1 mg/ml stocks and 1 µl was used per reaction. Although the Ct values for the uninoculated spleen DNA were lower than 40, they clearly did not increase with increasing concentrations of DNA. However, *Meq* detection in the inoculated spleen DNA rose significantly above this baseline when the DNA was used neat, or at 1:10 dilution. We thus established that, for Taqman analysis of DNA taken from tissue samples of MDV-inoculated chicks, we would use 1 µl DNA from a 1 mg/ml stock (ie. 1 µg) DNA per reaction.

Figure 6:

Time-course of replication of CVI988 vaccine in chicken feathers demonstrating peak replication 15-20 days post infection. This experiment was performed to follow the time-course of Rispens MDV-infection in feather axillary tracts of inoculated chicks by Taqman assay.

Taqman assay was used to detect the *Meq* gene in DNA prepared from feather tips of chicks at 0 (uninoculated), 10, 15, 20 and 28 dpi post inoculation with Rispens. A group of five chicks were sampled at each time-point (four chicks at 0 dpi). 1 µg DNA was used in the Taqman assay. Mean Ct values for each group are plotted. The Ct values decrease from 0 – 15 dpi, then increase again from 20 – 28 dpi, showing a peak of infection 15 – 20 dpi.

Figure 7:

This experiment was performed to follow the time-course of Rispens MDV-infection in feather axillary tracts of inoculated chicks by 132 bp repeat PCR.

DNA was prepared from feather tips of Rispens–inoculated chicks at 0 (uninoculated), 10, 15, 20 and 28 dpi. A group of five chicks were sampled at each time-point (four chicks at 0 dpi). 1 μg DNA was used in PCR (including 10 μg BSA per reaction) and the samples run on a 1% agarose gel containing ethidium bromide.

(M) = Lambda molecular size markers, (-) = water (negative control), (+) = Rispens BAC10 DNA (positive control). Days post infection are indicated underneath the gels.

(a) To confirm the PCR-quality and quantity of each DNA sample, PCR was performed to detect an endogenous retrovirus sequence present in all chicken cells. The 360 bp endogenous retrovirus product was detected in all of the feather samples confirming the PCR-quality of each samples.

(b) 132 bp repeat PCR was performed. The lane marked (+) shows the 132 bp repeat ladder PCR products obtained with Rispens BAC10 – six copies are clearly distinguished. The negative control shows no PCR product, as do three of the four uninoculated chicks. The fourth uninoculated chick shows a faint product band equivalent to 3 copies of the repeat, indicating that the chick was contact-infected by the inoculated chicks housed in the same room. All of the inoculated chicks were 132 bp repeat positive at 11, 15, 20 and 28 dpi. The PCR product representing a certain repeat copy number predominated in many cases and the number of copies represented by this predominant product varied between samples. This indicates that sub-clones of the inoculum virus, with a set number of repeats, come to predominate in different chicks.

Figure 8:

Graph showing a standard curve for *Meq* gene reaction using Rispens BAC10 DNA

Figure 9:

Graph showing a comparison of Rispens virus load in various feather tracts of individual chicks at (A) 8, (B) 13, (C) 19 and (D) 26 days post vaccination.

Figure 10:

Graph showing Rispens virus load in various feather tracts determined by real time PCR for Meq gene plotted as (A) on a logarithmic scale and (B) on a linear scale. Mean values (+SEM) for four vaccinated chicks.

EXAMPLE 1: quantitative PCR assay for MDV-1

Taqman™ quantitative PCR is an established technique used to quantify the amount of starting PCR target by determining the number of PCR cycles required to reach a fluorescence threshold (defined mathematically by the Ct value). A higher copy number of target sequence in the sample requires fewer PCR cycles to reach the Ct threshold.

The Taqman primers and probe were designed from the *Meq* gene sequence of the MDV strain GA (see later). This sequence is published in: Jones D, Lee L, Liu JL, Kung HJ, Tillotson JK. Marek disease virus encodes a basic-leucine zipper gene resembling the fos/jun oncogenes that is highly expressed in lymphoblastoid tumors. Proc Natl Acad Sci U S A. 1992 May 1;89(9):4042-6. The Sequence Accession No. is: M89471 (SEQ ID No. 9). The Applied Biosystems 'Primer Express' software was used to select the optimum primer/probe sequences from the Meq sequence.

The specific primers used in this analysis multiply a 73bp sequence in the meq gene of MDV serotype 1 virus. This region is common to both vaccine strains and field isolates.

The experiments described use CVI 988 vaccine as an example of serotype 1 Marek's disease virus. The results can be applied to all serotype 1 Marek's viruses because the area of the sequence the primers are directed against is conserved in MDV serotype 1 viruses.

Protocol for Taqman™ Meq gene PCR analysis on feather tip samples

1. Materials required:

Reagents:

- TNE buffer (store at room temperature) contains Tris (10 mM), NaCl (150 mM), EDTA (1 mM) and the pH is adjusted to pH 7.5 using HCl
- Sodium Dodecyl Sulphate (SDS) 10% solution (store at room temperature)
- Proteinase K (lyophilised powder from Sigma # P-6556, and make up a stock of 20 mg/ml in water, stored at –20°C)
- Phenol pH 7.9 obtained from Sigma (catalogue no. P-4557) stored at 4°C)
- Chloroform (stored at –20°C)
- 3M Sodium Acetate pH5.2 (stored at room temperature)
- Filtered neat ethanol (stored at room temperature)
- Filtered 70% cold ethanol (stored at 4°C)
- PCR quality water
- PCR quality water containing 800 µg/ml Bovine Serum Albumin (BSA), filtered
- Ice
- Taqman™ PCR core reagents – Taqman™ buffer, $MgCl_2$, dNTPs, Taq polymerase, Uracil N-Glycosylase (Perkin Elmer Biosystems)
  Meq forward primer 5' GGT CTG GTG GTT TCC AGG TGA 3' (SEQ ID No. 2) (MWG Biotech)
  Meq reverse primer 5' GCA TAG ACG ATG TGC TGC TGA 3' (SEQ ID No. 3) (MWG Biotech)
  Meq probe 5' FAM AGA CCC TGA TGA TCC GCA TTG CGA CT 3' (SEQ ID No. 1) TAMRA
  (Sigma-Genosys Ltd)
  (FAM & TAMRA are the fluorescent tags)

6-FAM = 6-carboxyfluorescein; (a phosphoramidite); yellow-green dye; absorbance maximum = 494 nm, emission maximum = 525nm
TAMRA = 6-carboxy-tetramethyl-rhodamine; absorbance maximum = 555, emission maximum = 580

5'FAM-3'TAMRA labelled probes are available from: Sigma-Genosys Ltd. (London Road, Pampisford, Cambridgeshire, CB2 4EF, UK. Tel. 01223 839200)

5'VIC-3'TAMRA labelled probes are available from: Applied Biosystems Ltd. (Kelvin Close, Birchwood Science Park North, Warrington, Cheshire, WA3 7PB)

Other suppliers are:

- Integrated DNA Technologies (IDT) 1710 Commercial Park, Coralville, IA, 52241, USA
- Oswel Research Products Ltd.: Lab 5005, Medical and Biological Sciences Building, University of Southampton, Boldrewood, Bassett Crescent East, Southampton, SO16 7PX, Tel: 02380 592984

Equipment

- Sterilin 20 ml plastic universal tubes
- Clean scissors & forceps
- Water bath set to 50°C
- Micro centrifuge
- 1.5 ml snap cap Eppendorf tubes (autoclaved)
- 1.5 ml screw-cap Eppendorf tubes (autoclaved)
  0.5 ml snap-cap Eppendorf tubes (autoclaved)

- Vacuum/freeze-drier (not essential)
- Spectrophotometer

  Dedicated PCR cabinet, pipettes and autoclaved tips
- Thermo-fast 96-well PCR plate and caps (Perkin Elmer/Applied Biosystems)
- ABI Prism 7700 Sequence Detector (Perkin Elmer/Applied Biosystems)

2. Collecting the feathers:

- Pluck 8 – 10 'pin' feathers (short, newly growing feathers with plenty of pulp) from the brachial feather tract of each chicken (see figures).
- Place feathers in a plastic 'universal' tube for transport back to the laboratory.

3. DNA preparation from feather tips:

- Cut off and save the proximal 1 cm of the feather (i.e. the non-barbed part which is attached to the skin and which contains the pulp – see photographs). Discard the distal barbed part of the feather.
- For each chicken, place the 8 – 10 saved feather ends in a 1.5 ml snap-cap Eppendorf tube.
- Add 500 µl proteinase K sample buffer (TNE buffer containing 0.5% SDS) containing 100 µg proteinase K (add proteinase K just before use).
- Incubate at 50°C in a water-bath for 1.5 – 2 hours.
- Microcentrifuge the tubes (6000 rpm, 10 min), to 'pellet' feather tips & debris.
- Transfer supernatant to a new snap-cap tube (if the feathers were from brown birds, the supernatants will be brown due to the presence of melanin).
- Add an equal volume (500 µl) of phenol to the supernatant again.

  Vortex

  Centrifuge at 13000 rpm, 2 min.

Transfer the upper (aqueous) phase to a new snap-cap tube.

- Add an equal volume (500 µl) of phenol to the supernatant again.
- Vortex
- Centrifuge at 13000 rpm, 2 min.
- Transfer the upper (aqueous) phase to a new snap-cap tube.
- Add an equal volume (500 µl) of cold chloroform.
- Vortex.
- Centrifuge at 13000 rpm, 2 min.
- Transfer the upper phase to a 1.5 ml screw-cap tube.
- Add 1 ml filtered 100% ethanol.
- Add 50 µl of 3M Sodium Acetate.
- Gently mix by inverting the tube, and leave at room temperature for 20 minutes (the DNA will become visible as it precipitates).
- Centrifuge 13000 rpm, 2 minutes, to pellet the DNA (if white chickens were used, pellet will be white; if brown chickens used, pellet brown).
- Discard the supernatant.
- Rinse the pellet twice with 500 µl of 70% cold ethanol, by gently running the ethanol down the side of the tube, then pouring off (take care not to dislodge the DNA pellet).
- Cover the open top of the tube with Parafilm, and make several puncture holes in the Parafilm using a needle.
- Place tubes in a vacuum drier for about 5 minutes to dry the pellet (alternatively air-dry).

Re-suspend the pellet in 50 µl PCR quality water by gently vortexing.

Determine the concentration of the DNA preparation using a spectrophotometer.

Adjust the concentration to 1 mg/ml in water.

Store at – 20°C.

4. *TaqMan™ quantitative PCR assay (Perkin Elmer Biosystems)*

- Set up reactions in a PCR-dedicated cabinet, using PCR-dedicated pipettes and autoclaved tips
- Work on ice
- Prepare master mix containing the following reagents for the appropriate number of samples – set up duplicate reactions for each sample (volumes given per reaction):

Component	Volume per 25 µl reaction	Final Concentration
Taqman buffer	2.5 µl	
$MgCl_2$ (5 mM)	5.0 µl	1.0 mM
dATP (10mM)	0.5 µl	0.2 mM
dCTP (10 mM)	0.5 µl	0.2 mM
dGTP (10mM)	0.5 µl	0.2 mM
dUTP (10mM)	0.5 µl	0.2 mM
Water containing 800 µg/ml BSA	11.6 µl	~10 µg BSA/reaction
Meq probe (10 µM)	0.5 µl	0.2 µM
Meq forward primer (10 µM)	1.0 µl	0.4 µM
Meq reverse primer ((10 µM)	1.0 µl	0.4 µM
Taq Gold DNA pol (5U/µl)	0.13 µl	26 U/ml
Uracil N-glycosylase (1U/µl)	0.25	10 U/ml

- Vortex to ensure complete mixing
- Place a thermo-fast PCR plate into a plate holder on ice and add 24 µl master mix to each well to be used
- Add 1 µl autoclaved water to no-template-control (NTC) wells and cap these wells prior to opening any DNA samples
- Add 1 µl positive control DNA (= 1 µl of 1 mg/ml preparation), eg. DNA from Rispens-infected CEF to appropriate wells and cap these wells

Add 1 μg sample DNA (= 1 μl of 1 mg/ml preparation), to appropriate wells and cap

Briefly pulse plate in centrifuge

- Place plate in ABI Prism 7700 Sequence Detector (Applied Biosystems) and set up computer to read FAM fluorescence (Meq probe), run samples
- *Thermocycling parameters*

50°C	2 min	
95°C	10 min	
94°C	15 sec)	x 40
60°C	1 min)	

- Analyse data using Microsoft excel – for each sample there will be a Ct value (the PCR cycle at which the amount of fluorescent product is first detected above baseline level); calculate mean Ct value for duplicates for each DNA sample.

Results of the above experiment are shown in Figure 6.

Notes

The Forward primer (GGT CTG GTG GTT TCC AGG TGA – SEQ ID No. 2) is located at NT positions 1341-1361 in the GA strain Meq gene sequence. The reverse primer (GCA TAG ACG ATG TGC TGC TGA – SEQ ID No. 3) is located at NT positions 1413-1393. The probe was designed to specifically anneal between the two primers on the Meq target sequence (see Figure 8).

During PCR, the fluorogenic probe binds between the two primers and, during each extension cycle, the 5' nuclease activity of the Taq polymerase cleaves the probe, releasing the reporter fluorochrome FAM that is then able

to fluoresce. Therefore, with each cycle, fluorescence intensity increases. The Ct value (the cycle at which fluorescence passes a fixed threshold) is a measure of the starting copy number of the target sequence: the higher the starting copy number, the lower the Ct value.

Use of Bovine Serum Albumin during PCR: feather tissues (especially those from brown chickens) contain melanin, which has been shown to be inhibitory to PCR. Use of BSA in the reaction overcomes this melanin-induced inhibition. Experiments were carried out in brown chickens. The addition of BSA followed the method of Giambernardi *et al* (1998) *Biotechniques* 25: 564-6.

EXAMPLE 2: Method for detecting specific MDV-1 (CV1 988 vaccine) strain

This example of the invention relates to the use of a specially modified version of the 132 base pair (bp) repeat polymerase chain reaction (PCR) test to detect the presence of CVI 988 Marek's vaccine in chicken feathers.

The 132 base pair repeat genetic sequence is located in the internal repeat long (IR_1) segment of the Marek's disease virus (serotype 1) genome. The complete genomic sequence of MDV 1 is described in Tulman *et al.* (Sept 2000) *J Virol.* Vol. 74 No. 17, p7980-7988 and has been deposited in GenBank under accession no. AF 243438. CV1 988 isolates (vaccine strains) of serotype 1 Marek's disease have multiple copies of this repeat segment, whilst field strains have single copies (Silva *et al* (1992) *Avian Dis* 36 : 521-528; and Becker *et al* (1993) *Virus Genes* 7 : 277-287). Measuring the number of copies affords the possibility of differentiating vaccine strains from field strains. (Becker *et al* (1992) *J Virol Methods* 40 : 307-322 and Kopacek *et al* (1993) *Acta Virol* 37 : 191-195).

Feathers are sampled according to the enclosed figures, the proximal tips of the axillary tract feathers being used. PCR analysis (see below) demonstrated multiple copies of the 132 bp segment in animals between 11 and 28 days post vaccination. Vaccination was carried out on birds at one day of age.

An acceptable variation of the test is to use feathers in birds of any age either taken as fresh samples, or stored for testing at a later date to determine the presence of the CV1988 vaccine.

Protocol for 132 bp repeat PCR on Feather tip samples

1. Materials required:

Reagents:

- TNE buffer (store at room temperature) contains Tris (10 mM), NaCl (150 mM), EDTA (1 mM) and the pH is adjusted to pH 7.5 using HC1
- Sodium Dodecyl Sulphate (SDS) 10% solution (store at room temperature)
- Proteinase K (lyophilised powder from Sigma # P-6556, and make up a stock of 20 mg/ml in water, stored at –20°C)
- Phenol pH 7.9 obtained from Sigma (catalogue no. P-4557) (stored at 4°C)
- Chloroform (stored at –20°C)
- 3M Sodium Acetate pH5.2 (stored at room temperature)
- Filtered neat ethanol (stored at room temperature)
- Filtered 70% cold ethanol (stored at 4°C)
- PCR quality water

PCR quality water containing 800 µg/ml Bovine Serum Albumin (BSA), filtered

Taq gold DNA polymerase (5 U/µl), Taq buffer, $MgCl_2$ (25 mM) from Bio/Gene Ltd, Kimbolton, Cambridgeshire, England

- dATP, dTTP, dCTP, dGTP (100 mM stocks) obtained from Promega (USA); we prepare a mix containing all four of these at 10 mM each, stored at – 20°C
- DNA molecular size markers
- Agarose and TBE buffer
- Primer sequences:

  MD-132 FOR; 5' TACTTCCTATATAGATTGAGACGT-3' (SEQ ID No. 4)

  MD-132 REV: 5' GAGATCCTCGTAAGGTGTAATATA-3' (SEQ ID No. 5)

Equipment:

- Sterilin 20 ml plastic universal tubes
- Clean scissors & forceps
- Water bath set to 50°C
- Micro centrifuge
- 1.5 ml snap cap Eppendorf tubes (autoclaved)
- 1.5 ml screw-cap Eppendorf tubes (autoclaved)
- 0.5 ml snap-cap Eppendorf tubes (autoclaved)
- Vacuum/freeze-drier (not essential)
- Spectrophotometer
- Dedicated PCR cabinet, pipettes & autoclaved tips
- Thermal cycler
- Agarose gel apparatus

2. <u>Collecting the feathers:</u>

- Pluck 8 – 10 'pin' feathers (short, newly growing feathers with plenty of pulp) from the axillary feather tract of each chicken (see figures).
- Place feathers in a plastic 'universal' tube for transport back to the laboratory.

3. DNA preparation from feather tips:

- Cut off and save the proximal 1 cm of the feather (ie the non-barbed part which is attached to the skin and which contains the pulp – see photographs). Discard the distal barbed part of the feather.
- For each chicken, place the 8 – 10 saved feather ends in a 1.5 ml snap-cap Eppendorf tube.
- Add 500 µl proteinase K sample buffer (TNE buffer containing 0.5% SDS) containing 100 µg proteinase K (add proteinase K just before use).
- Incubate at 50°C in a water-bath for 1.5 – 2 hours.
- Microcentrifuge the tubes (6000 rpm, 10 min), to 'pellet' feather tips and debris.
- Transfer supernatant to a new snap-cap tube (if the feathers were from brown birds, the supernatants will be brown due to the presence of melanin).
- Add an equal volume (500 µl) of phenol to the supernatant
- Vortex
- Centrifuge at 13000 rpm, 2 min
- Transfer the upper (aqueous) phase to a new snap-cap tube
- Add an equal volume (500 µl) of phenol to the supernatant again.
- Vortex
- Centrifuge at 13000 rpm, 2 min.
- Transfer the upper phase to a new snap-cap tube.
- Add an equal volume (500 µl) of cold chloroform.
- Vortex.

Centrifuge at 13000 rpm, 2 min.

- Transfer the upper phase to a 1.5 ml screw-cap tube.
  Add 1 ml filtered 100% ethanol.
- Add 50 µl of 3M Sodium Acetate.
- Gently mix by inverting the tube, and leave at room temperature for 20 minutes (the DNA will become visible as it precipitates).
- Centrifuge at 13000 rpm, 2 minutes, to pellet the DNA (if white chickens were used, pellet will be white; if brown chickens used, pellet brown).
- Discard the supernatant.
- Rinse the pellet twice with 500 µl of 70% cold ethanol, by gently running the ethanol down the side of the tube, then pouring off (take care not to dislodge the DNA pellet).
- Cover the open top of the tube with Parafilm, and make several puncture holes in the Parafilm using a needle.
- Place tubes in a vacuum drier for about 5 minutes to dry the pellet (alternatively air-dry).
- Re-suspend the pellet in 50 µl PCR quality water by gentle vortexing.
- Determine the concentration of the DNA preparation using a spectrophotometer.
- Adjust the concentration to 1 mg/ml in water.
- Store at – 20°C

4. <u>132 bp repeat PCR:</u>

- Set up reactions on ice, in 0.5 ml Eppendorf tubes, in a PCR-dedicated cabinet, using PCR-dedicated pipettes and autoclaved tips.
- Prepare a 'master mix' containing the following reagents for the appropriate number of samples (volumes given per reaction):

Component	Volume per 20 µl reaction	Final Concentration
Forward primer (10 µM)	1.0 µl	0.5 µM
Reverse primer(10 µM)	1.0 µl	0.5 µM
10 x Taq buffer	2.0 µl	
$MgCl_2$ (25 mM)	1.6 µl	2 mM
DNTP mix (10 mM)	0.5 µl	0.25 mM
Taq gold DNA polymerase	0.1 µl	25 units/ml
Water containing 800 µg/ml BSA*	12.8 µl	10 µg BSA/reaction

- Vortex to ensure complete mixing.
- Aliquot 19 µl 'master mix' into autoclaved 0.5 ml snap-cap Eppendorf tubes.
- Add 1 µg DNA (= 1 µl of 1 mg/ml preparation, or larger volume if DNA preparation less concentrated).
- Vortex to ensure complete mixing.
- (Our thermal cycler has a heated lid, so we do not need to overlay the reactions with mineral oil).
- Run on a thermal cycler using the following cycling parameters:

95°C	2 min	1 cycle
95°C	1 min)	
50°C	30 sec)	x 40 cycles
72°C	1 min)	
72°C	10 min	1 cycle

- Analyse reaction products on an agarose gel.

The results of the above experiment are shown in Figure 7.

Notes

The sense primer is located 65 bp upstream of the repeat and the antisense primer is located 105 bp downstream of the repeat. The expected band size

is therefore 302 bp for a single repeat (i.e. 65 + 132 + 105), 434 bp for a double repeat (302 + 132) and 566 bp for a triple repeat (434 + 132) etc. Rispens vaccine strain produces many tandem repeats.

EXAMPLE 3 – Detection of Rispens virus genome in feather tip DNA using PCR: comparison of samples from different feather tracts.

Methods

Feather sampling from Rispens-vaccinated chicks

Two-week-old Rhode Island Red chicks were inoculated with 1000 pfu Fort Dodge Rispens vaccine virus via the intra-peritoneal route. Age-matched, non-vaccinated chicks were housed in a separate room. All chicks were wing-banded in both wings, to permit identification of individual chicks throughout the experiment. At 8, 13, 19 and 26 days post vaccination, five vaccinated chicks (#921, #923, #924, #925) and one non-vaccinated chick (#946) were sampled. Approximately six pinfeathers were plucked from each of the Cervical, Humeral, Spinal, Axillary and Femoral tracts. The remaining five feather tracts were not used either because the number of feathers was too few to allow sampling on four occasions, or because it was considered unethical to pluck from delicate regions of the skin of living chicks.

DNA was prepared from the feather tips and subjected to real-time PCR to detect the virus *Meq* gene, as summarised below.

DNA preparation from feather tip samples

DNA was prepared from feather tips as described above. Briefly, feather tips were incubated at 50^{0}C for 2 hours in 500 µl TNE-SDS buffer containing 100 µg proteinase K. The supernatant was extracted with

phenol, then with ice-cold chloroform. DNA was precipitated, using ethanol/sodium acetate, pelletted, vacuum dried, then resuspended to a concentration of 200 µg/ml in water.

Real-time (TaqMan) PCR assay to detect Meq gene

25 µl duplex reactions were set up as described above, using *Taq* gold DNA polymerase and primers to amplify the viral *Meq* gene and the host Ovotransferrin (*Ovo*) gene (table 1) from 200 ng feather tip DNA. BSA was present at a concentration of 10 µg per reaction. The reaction kinetics were followed by inclusion of a FAM-fluorescent-tagged *Meq* probe and a VIC-fluorescent-tagged *Ovo* probe (table 1). There were duplicate reactions for each sample. The thermocycling parameters were: 1 cycle of 50^{0}C (2 min), 1 cycle of 95^{0}C (10 min), followed by 40 cycles of 94^{0}C (15 sec) and 60^{0}C (1 min). In addition to the feather samples under test, the reaction plate also included ten-fold dilutions of Rispens BAC10 DNA (Rispens virus genome cloned into a Bacterial Artificial Chromosome), containing a calculated number of copies of the Rispens genome.

The data were analysed using Microsoft Excel. For each reaction, the Ct value (the PCR cycle at which the amount of fluorescent product is first detected above baseline level), and thence the 40-Ct value, was obtained. A Ct value of 40 indicates that no target sequence was present. Values are presented as '40 – Ct' so that a higher value equates to more copies of the viral genome.

For the Rispens BAC10 samples, 40-Ct value was plotted against number of copies of virus genome (log scale), to produce a standard curve. The equation for the linear portion of the plot was determined. For the feather samples under test, the 40-Ct values were 'normalised' according to the 40-Ct value for *Ovo* (a 'house-keeping gene' present in all chicken cells), to

correct for slight differences in the amount of DNA used for each sample. The mean, normalised 40-Ct value for duplicate reactions was then determined and was converted to Rispens genome copy number using the standard curve.

Table 1: Primers and probes used in conventional and real-time PCR

Target Sequence	Primer Name	Primer Sequence (5' – 3')	Primer Location	Amplicon Size
MDV-1 *Meq* gene (based on GA sequence)	Meq forward	GGTCTGGTGGTTTCCA GGTGA (SEQ ID No. 2)	1341–1361	73 bp
	Meq reverse	GCATAGACGATGTGCT GCTGA (SEQ ID No. 3)	1413-1393	
	Meq probe	AGACCCTGATGATCCG CATTGCGACT (5' FAM label, 3' TAMRA label) (SEQ ID No. 1)	1366–1391	
Ovotransferrin gene (Ovo)	Ovo forward	CACTGCCACTGGGCTC TGT (SEQ ID No. 6)	4517-4535	62 bp
	Ovo reverse	GCAATGGCAATAAAC CTCCAA (SEQ ID No. 7)	4567-4587	
	Ovo probe	AGTCTGGAGAAGTCT GTGCAGCCTCCA (5' VIC label, 3' TAMRA label) (SEQ ID No. 8)	4537-44564	

Results

Feather sampling and DNA preparation

Although it was initially planned to sample each of the ten feather tracts, it was only practicable to use the Cervical, Humeral, Spinal, Axillary and Femoral tracts. The remaining five feather tracts could not be used either because the number of feathers was too few to allow sampling on four occasions, or because it was considered unethical to pluck from delicate regions of the skin of living chicks. Sufficient pinfeathers were obtained from each tract at 8, 13 and 19 days post vaccination. By 26 days (when the chicks were almost 6 weeks old) the pinfeathers were beginning to be replaced by harder, mature feathers. Sufficient DNA was obtained from all samples, with no marked differences between amount of DNA obtained from the same number of feathers from different tracts. In order that the *Meq* and *Ovo* Ct values fell into the linear range of detection for real-time PCR, it was found necessary to dilute the DNAs to a concentration of 200 ng/µl and to use 1 µg of this stock per for real-time PCR reactions.

To enable accurate comparison of virus load at different time-points, in different feather tracts, and in different chicks, real-time quantitative PCR was performed.

For each reaction, the 40-Ct value was calculated. For the Rispens BAC10 samples, 40-Ct value was plotted against number of copies of virus genome (log scale), to produce a standard curve (Figure 8). The lower limit of accurate detection was 50 copies thus, any samples with a 40-Ct value of 0 contain fewer than 50 copies of the Rispens genome per 200 ng feather tip DNA.

The equation for the linear portion of the plot was: $y = 1.8102 \text{ Ln}(X) - 6.796$. For each of the feather samples under test, the mean, normalised 40-Ct values were converted to Rispens genome copy number using this equation (see Tables 2A, B, C and D; Figure 9 A, B, C and D). As expected, there was considerable variation between individual chicks at any given time-point. However, the levels of virus detected in the five different feather tracts of a given individual were similar. In each chick, of the four timepoints tested, virus load was greatest at 13 days, decreasing at 19 and 26 days.

Table 2A: Rispens MDV copy number (8 days)

8 days post vaccination					
Chick # & feather tract	Copies of Rispens MDV genome / 200ng feather DNA				
	Humeral	Spinal	Femoral	Cervical	Axillary
#921	673	537	2630	442	778
#923	1504	4890	5127	5921	6101
#924	112910	61242	258345	31425	62444
#925	2603	1265	2010	1883	1583
Mean	29422	16983	67028	9918	17726
SDev	55664	29567	127552	14524	29904
SE mean	27832	14784	63776	7262	14952

Table 2B: Rispens MDV copy number (13 days)

13 days post vaccination					
Chick # & feather tract	Copies of Rispens MDV genome / 200ng feather DNA				
	Humeral	Spinal	Femoral	Cervical	Axillary
#921	2133045	2564484	2432204	1224616	2511886
#923	3814060	4374639	5998652	3561896	38349161
#924	24768376	876646	4241591	24073508	24711780
#925	866276	4478465	1557400	1856729	485447
Mean	7895439	3073559	3557462	7679187	16514568
SDev	11313237	1708035	1974326	10974035	18229569
SE mean	5656619	854018	987163	5487018	9114785

Table 2C: Rispens MDV copy number (19 days)

19 days post vaccination					
Chick # & feather tract	Copies of Rispens MDV genome / 200ng feather DNA				
	Humeral	Spinal	Femoral	Cervical	Axillary
#921	353997	613762	1158777	736207	439542
#923	1040917	1427067	1061134	1029830	1335442
#924	3108135	891103	256243	1584386	391420
#925	2958332	9800358	1469708	3373701	1705109
Mean	1865345	3183073	986466	1681031	967878
SDev	1378768	4424421	517048	1181980	655758
SE mean	689384	2212211	258524	590990	327879

Table 2D: Rispens MDV copy number (26 days)

26 days post vaccination					
Chick # & feather tract	Copies of Rispens MDV genome / 200ng feather DNA				
	Humeral	Spinal	Femoral	Cervical	Axillary
#921	295801	193642	167109	74645	95940
#923	921896	694141	541176	976157	569305
#924	771634	615826	349598	343195	104585
#925	353768	926750	511690	251692	236766
Mean	585775	607590	392393	411422	251649
SDev	308455	305928	172194	392646	221359
SE mean	154228	152964	86097	196323	110680

For the four vaccinated chicks, the mean genome copy number (per 200 ng feather DNA) was determined for each feather tract at each time-point, and plotted against time post vaccination using either a logarithmic scale (Figure 5) or a linear scale (Figure 10B). At 8 days, mean copy number was between 10^4 and 10^5 copies per 200 ng DNA. At 13 days, mean copy number was between $5x10^6$ and $5x10^7$; at 19 days, between 10^6 and 10^7; and at 26 days, between 10^5 and 10^6.

The logarithmic plot (Figure 10A) indicated similar virus replication kinetics in each of the five feather tracts. However, differences are emphasised in linear plot (Figure 10B). This plot shows that, at 13 days, the mean virus load for four chicks is up to four-fold greater in the axillary tracts than in the other four tracts. At this time-point, virus loads in the humeral and cervical tracts were very closely related at each time-point, and were greater than virus load in the femoral and spinal tracts. These differences between feather tracts were not apparent at 8, 19 or 26 days.

For the non-vaccinated control chick (#946) the majority of the samples gave 40-Ct values of 0, as would be expected in the absence of MDV DNA.

However, for some of the samples, the 40-Ct values were up to 3 (data not shown). It is often the case in real-time PCR that 'negative' samples give a 40-Ct value above 0, and values smaller than 4 are considered 'unreliable'. Thus, although a 40-Ct value of 3 equates to ~200 copies of Rispens genome, this value is 100-fold lower than any values obtained for samples from vaccinated chicks, and can be considered negative. As seen in figure 5B, the mean copy number for the five feather tracts of the non-vaccinated chick does not rise above the baseline.

Summary of real-time PCR analysis

- Virus load in each of the feather tracts was sufficient to be detectable by real-time PCR at 8, 13, 19 and 26 days post vaccination.
- Virus load was highest in all of the tested feather tracts at 13 days post vaccination, as compared with 8, 19 and 26 days post-vaccination.
- At 13 days post vaccination, virus load was higher in the axillary tract than in the humeral and cervical tracts (two times greater) or in the spinal and femoral tracts (four times greater).